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13. ABSTRACT (Maximum 200) Genomic amplification is believed to be a pivotal event in the origin and progression of a variety of human tumors including breast cancers. Recently, a technical innovation potentially suitable for detecting candidate novel amplifications in tumors, called restriction landmark genomic scanning, has been described by Hyashizaki and co-workers. This is a two-dimensional DNA restriction analysis technique by which a highly reproducible constellation of ~2,000 spots (each corresponding to a specific genetic locus) can be surveyed. As spot intensity is proportional to gene copy number in this system, amplified loci in tumor-derived DNAs are readily detected. Using this approach, several investigators including ourselves have demonstrated both previously identified and novel amplicons in several tumor types. A derivative technology allows direct cloning of amplified sequences from DNA isolated from excised spots. I propose here to study up to 20 breast tumor specimens in this way. Candidate novel amplicons identified will be cloned; initial characterization of these amplicons will entail their genomic localization using fluorescence <i>in situ</i> hybridization.				
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FOREWORD

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INTRODUCTION

Several distinct types of genetic alterations are believed to be causal in the formation and progression of tumors (1, 2). Gene amplification is a prominent molecular lesion in a variety of human tumors, including breast cancers (1-8). By increasing the copy number of a gene through amplification, high level expression of the corresponding gene product may result; where the gene is a classical dominant oncogene that positively regulates cell growth, pathological dysregulation of proliferation that contributes to malignancy can ensue (1, 2). Gene amplification *per se* may also be a reflection of a state of "genomic instability" characteristic of neoplastic cells (4).

Amplification of several growth control-associated genes in carcinomas of the breast has been observed at various frequencies (reviewed in (3, 8)); these include the growth factor receptor genes ERBB2, EGFR, IGF-IR, and FGF receptors 1, 2 and 4; the proto-oncogenes CMYC and CYCD/ BCL1/ PRAD1; and the p53-binding protein MDM2. [Although amplification of the genes for the growth factors FGF3 and 4 has also been observed in breast carcinomas, these are not thought to be significant in pathogenesis; instead, these are believed to reflect passive amplification of the genes "driven" by the adjacent CYCD locus (see (8))].

Gene amplification in tumors has been detected most frequently by the "shotgun" approach of DNA blot hybridization to a battery of cloned oncogene probes, sometimes guided by prior biochemical or immunoassay data. This approach suffers the drawback that amplification of previously unknown sequences can not be detected in this way. Rarely, amplified novel oncogenes have been discovered when present in tumors in characteristic cytogenetic structures (9, 10). In general, however, identification of novel amplified sequences in tumors has been problematic.

A rather recently described technique, comparative genomic hybridization (CGH), has offered a new approach to detecting genetic amplification (and deletion) in tumors (11). Briefly, this is a two-color fluorescence *in situ* hybridization (FISH) method that allows relative gene dosages to be assessed on a genome-wide basis; detection is based on the relative kinetics of reannealing of differently-colored tumor-derived and normal cell-derived fluorescent DNA probes to normal metaphase spreads. A relative excess or deficiency of a particular gene sequences in a tumor results in a brighter tumor-specific or normal cell-specific signal at the corresponding chromosomal locations on the metaphases, respectively. Preliminary CGH studies of breast cancer specimens have demonstrated candidate novel (distinct from ones noted above) genomic amplifications (12, 13, 14). CGH does not permit direct isolation of genomic sequences that have been found to be amplified.

BODY

1. Experimental Strategy and Methods

Restriction landmark genomic scanning (RLGS; "genomic scanning" for short) is a genetic analysis tool developed by Hayashizaki and coworkers (5, 6, 15, 16). In RLGS, mammalian (or in some instances, yeast) DNA is first cut using a restriction enzyme (such as Not I or Asc I) of the special class that cuts relatively infrequently (i.e., ~10,000 times in the 3 billion basepair haploid human genome); the ends so generated are specifically radiolabeled using a DNA polymerase to fill-in the ends, and the reaction is heat-killed. The DNA is then

cut with a second enzyme, such as Eco RV or PvuII, that cuts quite frequently. The cut-and-labeled DNA is next resolved according to molecular length for the first time using agarose tube gel electrophoresis. A third restriction enzyme (such as Hinf I or PstI) is then diffused into the agarose tube gel in order to cut the DNA a third time *in situ*. The DNA is next resolved by length a second time using a polyacrylamide slab gel. The end-radiolabeled DNA is visualized by autoradiographic exposure of X-ray film. A highly reproducible pattern of "spots" is observed which is characteristic of the species under study and the combination of restriction enzymes employed; each spot corresponds to one genomic location. Typically ~2,000 spots are well-resolved when the gels are cut into easily handled pieces; this corresponds to a sampling of the genome at *ca.* 1 megabase pair intervals on average. The intensity of each spot is proportional to the number of gene copies present in the cell of the corresponding genomic region.

The majority of spots in human DNA are 2-copy, because two identical alleles are present at the genomic locus of origin (7). A few highly reproducible spots in normal mammalian DNA are of greater than two copy intensity; detailed characterization of these by sequence database analysis and cloning has shown that they represent primarily ribosomal RNA genes (17). About 10% of human spots are 1-copy due to the presence of polymorphic alleles (e.g., each allele makes a separate 1-copy spot) (7, 18). It has been found that RLGS can be a useful tool for the identification of genetic amplification and deletion events associated with tumorigenesis (19, 20). Comparison of normal and tumor DNA from neuroblastoma patients, for example, has revealed amplification of the N-myc gene and of an additional novel locus in two patients not having N-myc amplification (19). Genetic deletion events, possibly corresponding to tumor suppressor gene deletions, are commonly observed in tumor DNAs (15, 19).

2. Results and Discussion

a. RLGS analysis of human breast carcinomas

We have proposed to use RLGS as an approach to 1. identify, and 2. clone, candidate novel genomic amplifications in human breast carcinoma (HBC); the ultimate goal of the proposed studies is to isolate new dominant oncogenes significant in the pathogenesis of HBC. To this end, in year 1 of support we surveyed fifteen HBC cell lines and primary tumor specimens derived at the University of Michigan Health System, as detailed in the proposal and statement of work; these specimens had been pre-screened by Southern blotting to exclude the "major" amplicons in HBC (see above). We have not found evidence for novel amplicons in any of these materials. This result is consistent with similar studies that we have conducted on human malignant gliomas (Radany, et al., unpublished results) in which a candidate novel amplification (as opposed to previously characterized ones) was detected in only one of 23 specimens that had not been pre-screened by CGH (see below). We did find evidence of an unusual apparent demethylation (19) of two of the dominant ribosomal RNA gene-associated spots (17) (Figure 1) in three of the HBC specimens. The significance of this result is uncertain; demethylation of repetitive elements in tumors, detected by RLGS, has been reported previously (19). Since this finding is unrelated to the goal of detecting novel amplicons in HBC, it is not being pursued at this time.

We can offer several reasonable, and non-mutually exclusive, explanations for the failure to find novel amplifications in the specimens examined to date: 1. these specimens simply did not contain any amplicons (in the case of the cell lines studied, this could possibly be a function of the biology of those tumors that are able to be established in culture); 2. novel amplicons most frequently co-exist with known ones (like ERBB2) and so any such specimens would have been excluded by the Southern blot pre-screening; and 3. the current RLGS protocol is not sufficiently sensitive to detect novel amplicons in this relatively small collection of tumors.

In the face of such results for HBC and other tumors, in our hands and those of colleagues, since submission of the proposal we have come to regard RLGS as a cloning tool that is optimally utilized in conjunction with specimen pre-screening by CGH. In this way, RLGS resources can be devoted exclusively to the characterization of those tumor specimens that contain novel amplicons; in this setting, it is feasible to do the RLGS assays more intensively (that is, utilize more different combinations of restriction enzymes for the analysis) than when RLGS is used as the primary survey tool. Furthermore, tumors containing extant amplicons need not be excluded from the analysis (if it has been shown that they contain novel ones as well). These features increase the sensitivity of the individual assays, and of the overall studies, compared to the HBC experiments done in Year 1. WE ARE INCORPORATING THIS REVISED, CGH-ASSISTED STRATEGY IN TWO WAYS DURING THE SECOND YEAR OF THE STUDY (see: Recommendations).

b. An improved "spot" cloning method

While it has proved possible to directly clone amplified DNA sequences out of material extracted from excised gel "spots" in several cases, using conventional plasmid vectors and electroporation-competent *E. coli* cells (19), we have not been uniformly successful in this approach. As the goal of Technical Objective 2 is to clone the DNA sequences corresponding to spots that derive from candidate novel amplicons in HBC, during Year 1 we experimented with alternative cloning approaches that might be more consistently successful; we focused on a PCR-based strategy (See--Figures 2 and 3).

We have found that it is feasible to combine an affinity purification step for the initial NotI-ended genomic fragments with the ligation of a specific oligonucleotide "tag" (the so-called SP6 promoter universal primer). This tag makes it possible to extensively purify, subsequently, a NotI genomic fragment of interest isolated from a gel spot and thereby remove the large excess of HinfI-HinfI fragments that are present. This step is done following ligation of a Hinf I-ended amplimer oligonucleotide. In this way, a final PCR amplification step using SP6 and the Hinf I amplimers yields a product that can be efficiently cloned or [in the case of spots from gels of yeast artificial chromosome (YAC)- containing yeast hosts] sequenced directly (see below). A manuscript describing this approach is in preparation.

c. Combined RLGS genomic analysis using tumor and YAC DNAs

The resources of the Human Genome Project are of potential value in the proposed experiments. We investigated the feasibility of combining YAC contigs and the corresponding physical and genetic map information with analysis of tumor amplicons using RLGS. In a pilot study, amplification the EGFR locus in human tumors was found to give rise to a

constellation of high intensity spots (three of these, designated #s 3 ->5, are shown in Figure 4). We demonstrated that these spots could be ordered on the human physical and genetic maps by RLGS analysis of several YACS from the human chromosome 7p12 region. The nucleotide sequence of spot #5 was determined directly by PCR amplification as noted above. A manuscript describing this work is in preparation.

3. Recommendations--Revised plans for Year 2.

Through the experience of both ourselves (in regard to studies on human gliomas) and several of our colleagues (who study a variety of tumors) we have come to believe that RLGS is best used in conjunction with CGH pre-screening of tumor specimens to find ones that contain novel amplifications. Such tumors may, in turn, be studied with many combinations of restriction enzymes for the RLGS to identify a maximal number of amplicon-derived spots; the latter may then be cloned to derive amplicon probes. Our collaborator, Steven Ethier, Ph.D., of this institution, has recently completed a collaborative CGH analysis of many breast tumor cell lines derived by his research group. Four of these contain a novel amplicon at chromosome 8q. In conjunction with Dr. Ethier (letter appended) we will analyze these four cell lines using RLGS with up to ten enzyme combinations in an effort to detect and clone spots derived from this genomic locus. Dr. Samir Hanash of this institution, who is a collaborator with us on RLGS studies unrelated to the goals of this grant, has found evidence of another novel amplicon in HBC on chromosome 17 q in the vicinity of the gene TBX2 (21); YACs are available for this region (21). In the light of our developed expertise in RLGS analysis of YACs, we are currently analyzing this contig in an effort to derive probes from this region as well.

CONCLUSIONS are deferred pending completion of the study in Year 2.

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LIST OF APPENDICIES

Appendix 1--Figure legends and Figures

Appendix 2--Letter of collaboration, Dr. S. Ethier

Appendix 1--Figure Legends and Figures

Figure Legends

Figure 1. Novel demethylation of ribosomal RNA genes in human breast carcinoma. Top: Portion of an RLGS gel from a human breast carcinoma cell line showing three novel high intensity spots (arrows) believed to represent demethylation of typical ribosomal RNA gene sequences (high intensity spots without arrows). Bottom: Normal cell DNA for comparison.

Figure 2. Affinity purification and tagging of *NotI*-ended genomic fragments. Page 1 Top: *NotI* + *EcoRV* cut genomic DNA (right) is mixed and ligated (middle) with a biotinylated duplex oligonucleotide having a *NotI* compatible cohesive end, an internal *NotI* site, and the SP6 promoter sequence as a tag (top left). The tagged genomic *NotI*-ended fragments are captured using avidin magnetic beads (bottom). Page 2: The captured *NotI*-ended genomic fragments are recovered magnetically (Top) and then freed from the beads by *NotI* cutting (Bottom). The tagged affinity purified fragments are then run on a 2-D gel in the usual way.

Figure 3. Selective capture + PCR amplification of *NotI*-*HinfI* fragments isolated from gel spots. An amplifier oligonucleotide is ligated to the *HinfI* ends of the mixed population of DNA molecules isolated from a gel spot. The *NotI*-ended fragments are isolated selectively via a biotinylated complimentary oligonucleotide and magnetic avidin beads. The material so captures is next PCR-amplified with SP6 tag and *HinfI* amplimers. The PCR products are then cloned or sequenced directly.

Figure 4. Confirmation of YAC spot identifies by 2-D gel analysis of mixed YAC and tumor DNAs. Left: YAC-specific spots in yeast genomic DNA are indicated as open arrows, while host background spots are indicated as filled arrows; YAC spots candidate for ones corresponding to 7p12 amplicon-associated spots are indicated by numbers. Right: Mixing of YAC and tumor DNAs for 2D analysis confirms exact identity of electrophoretic mobilities of YAC and tumor amplicon-associated spots.

Figure 1

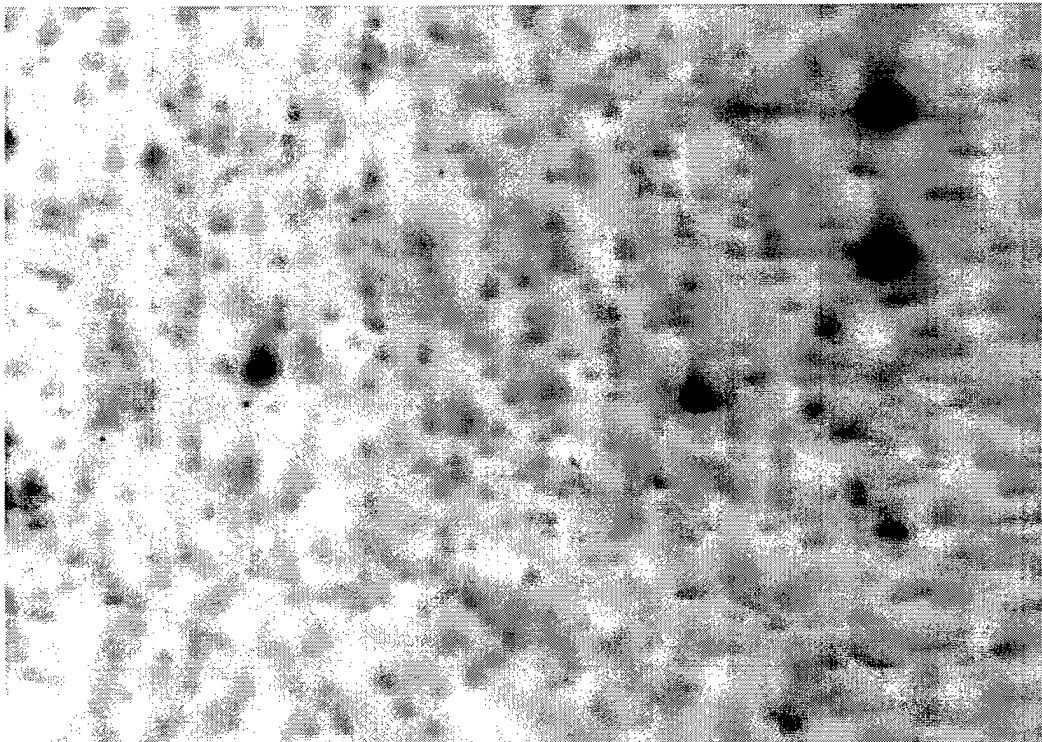
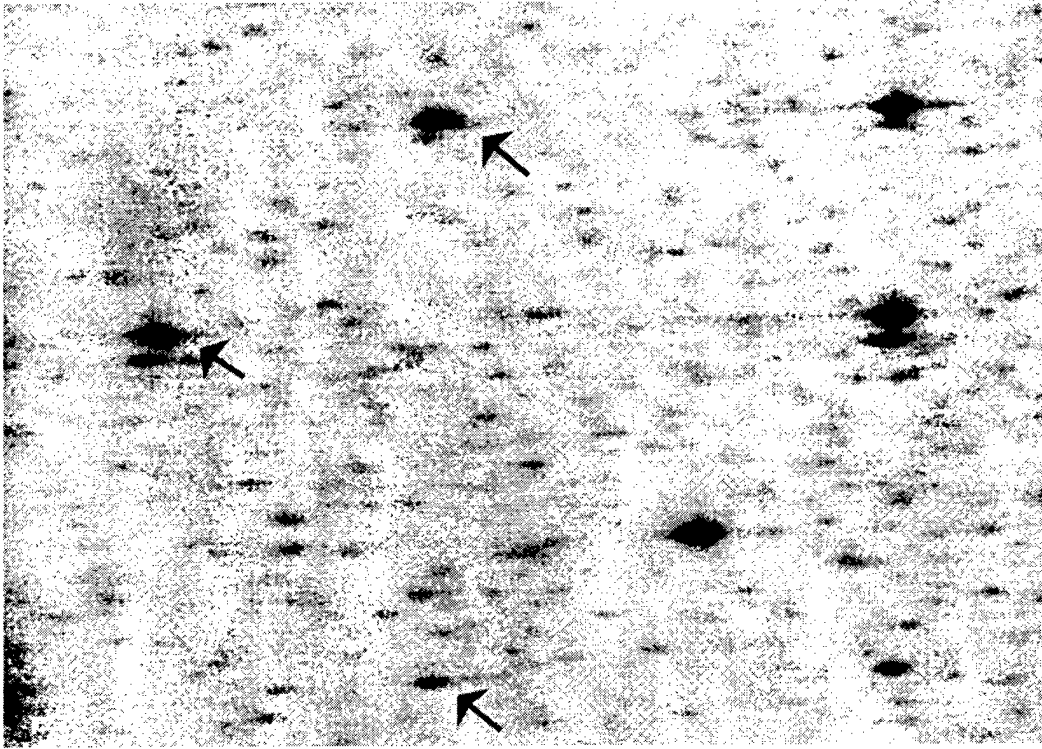


Fig. 2 Affinity purification and "tagging" of NotI-ended genomic DNA fragments

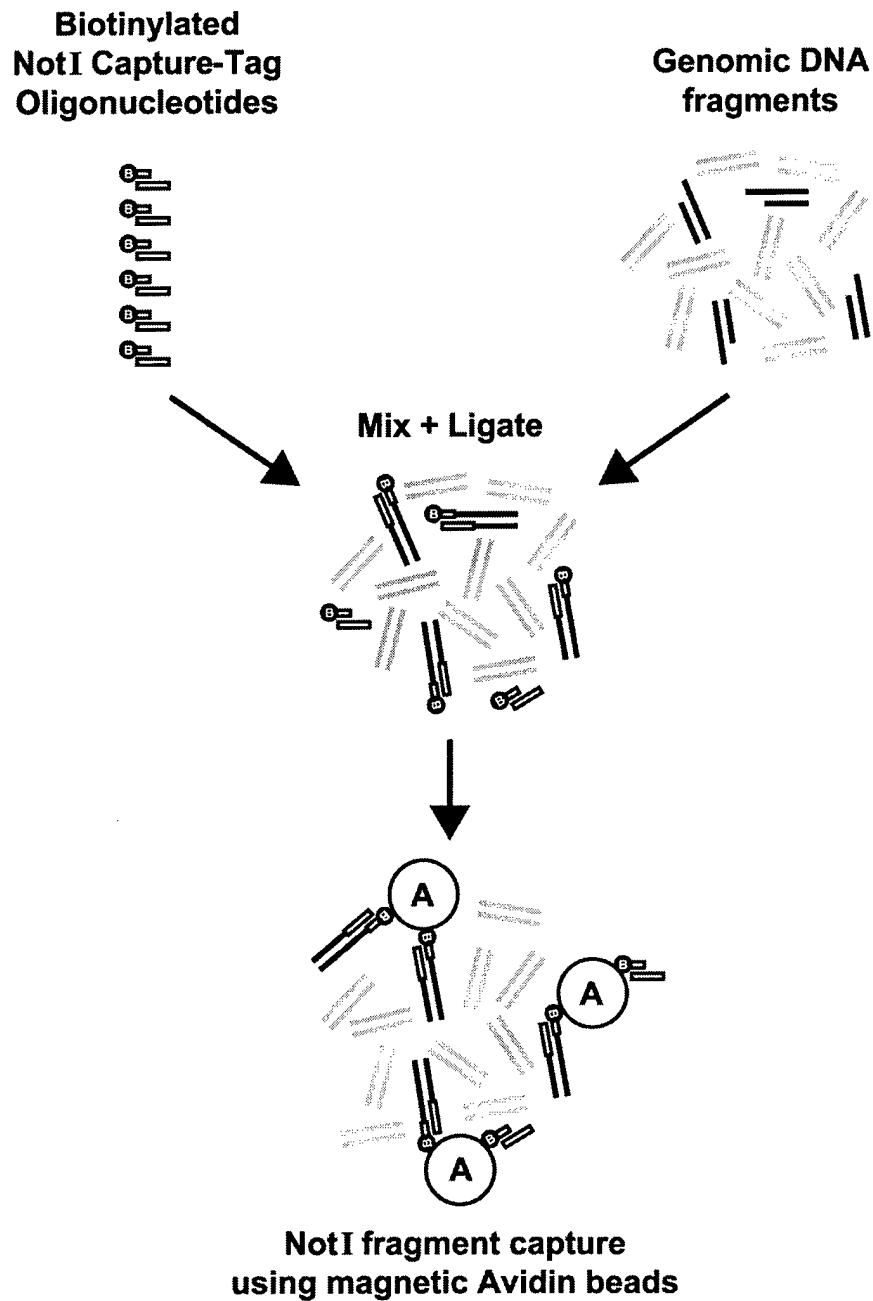


Fig. 2 (continued)

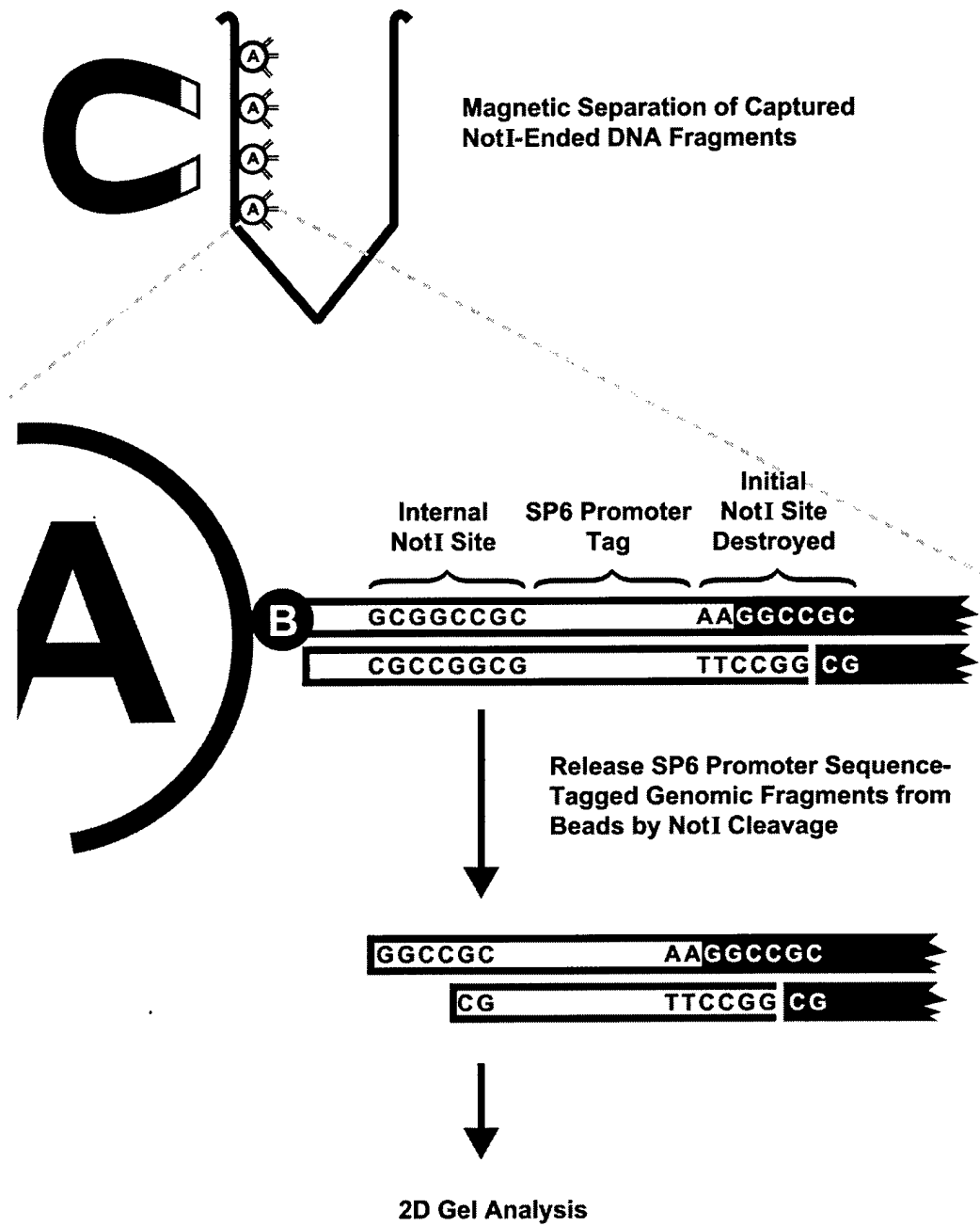


Fig. 3 Selective capture and PCR amplification of NotI-HinfI fragments isolated from gel spots.

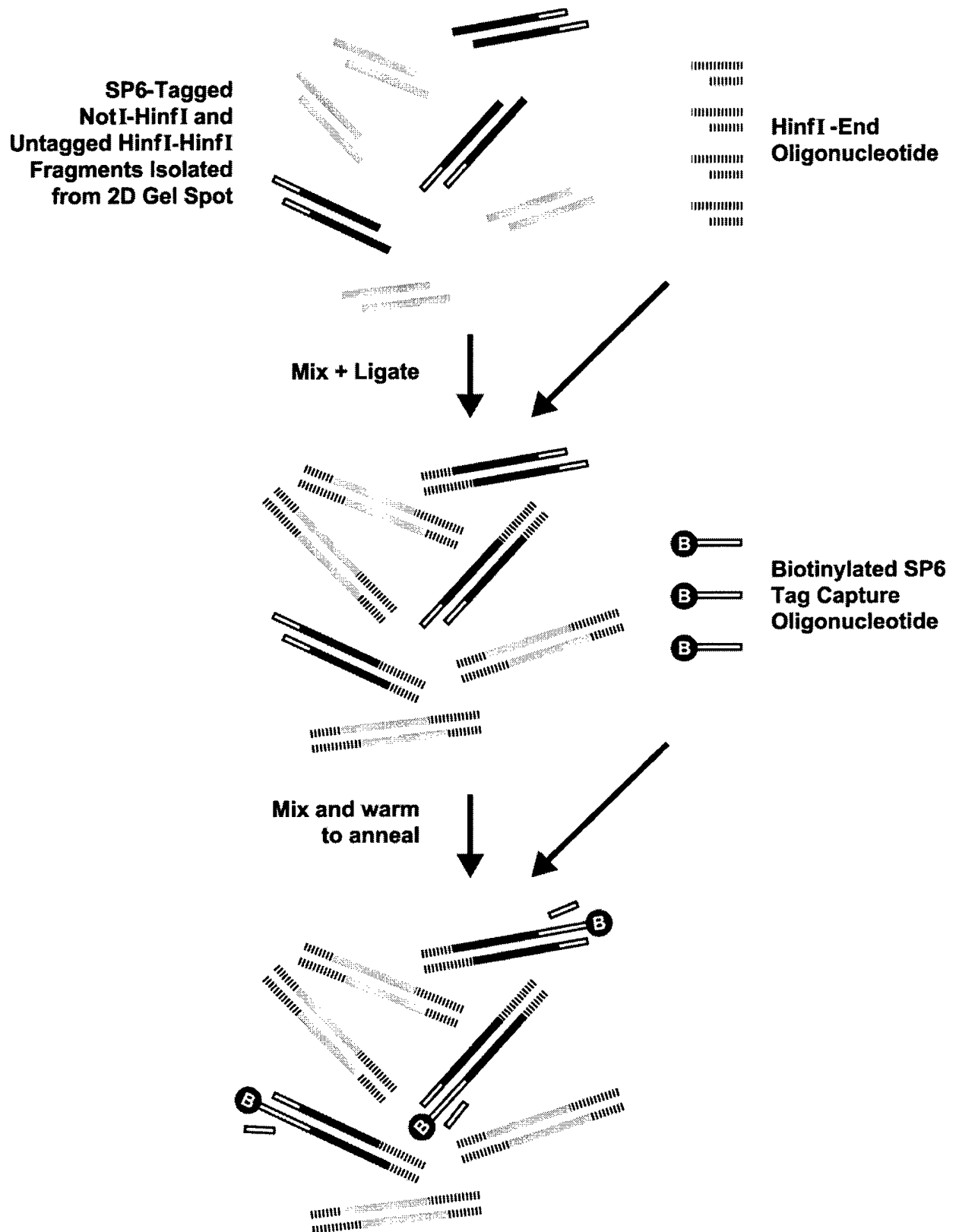


Fig. 3 (continued)

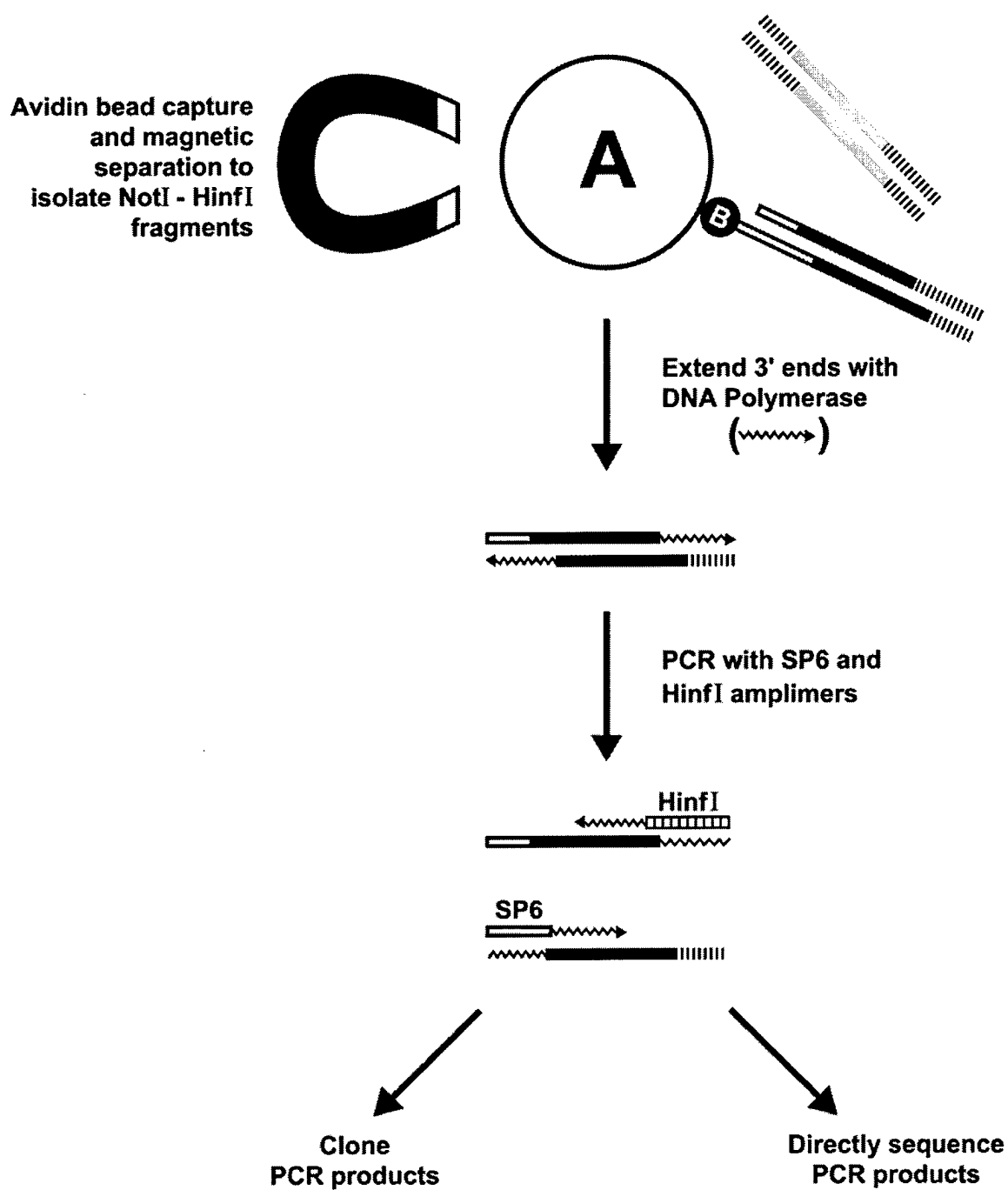
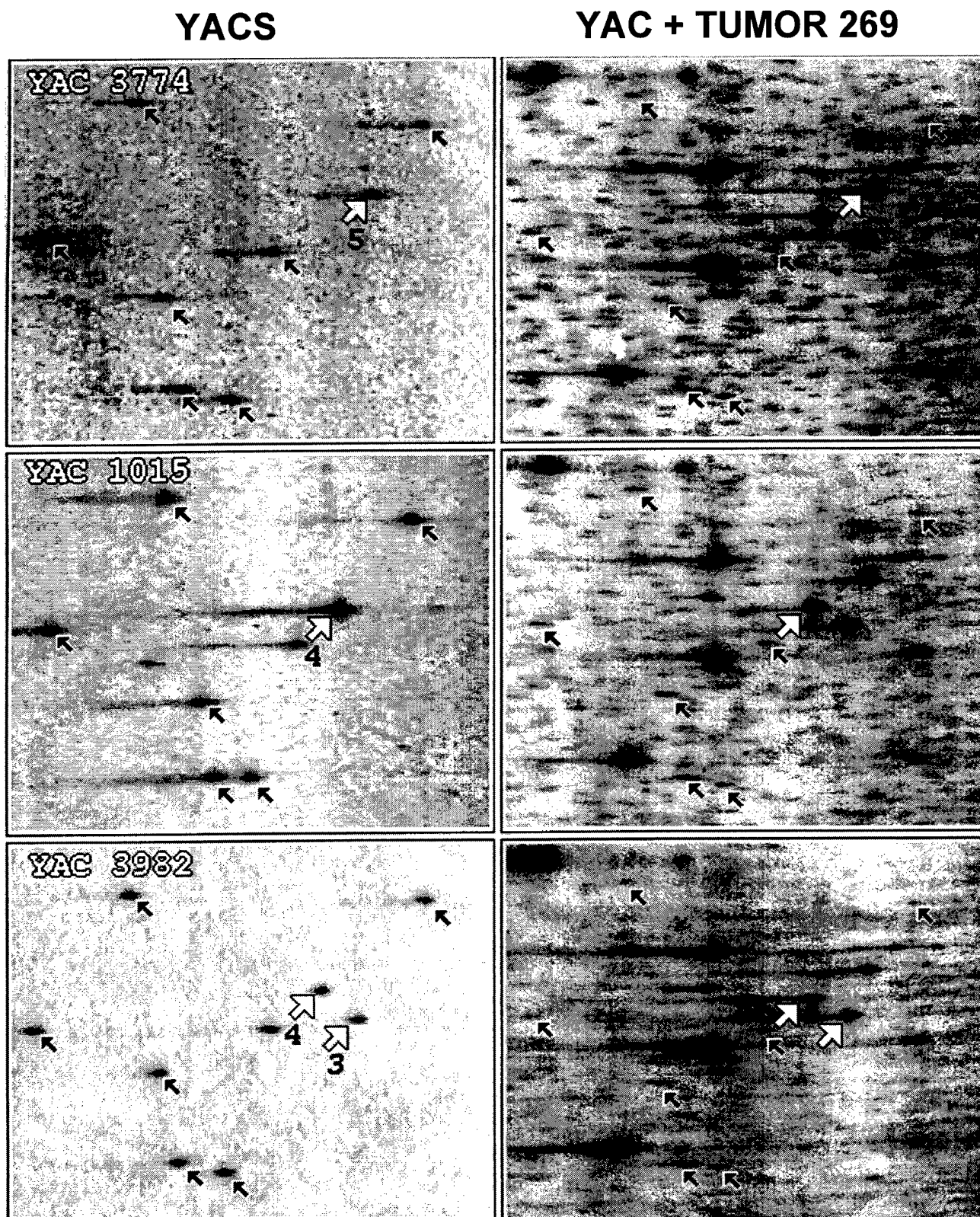


Fig. 4 Confirmation of YAC spot identities by 2-D gel analysis of mixed YAC and tumor DNAs.





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January 20, 1998

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Dear Eric:

This letter is to document, for the purpose of your Year 1 progress report to the DOD Breast Cancer Program, my willingness to supply you with a number of human breast cancer cell lines that have been studied by comparative genomic hybridization. As you know, some of these lines show evidence of a novel amplicon on chromosome 8. The recurrence of this finding in multiple samples certainly suggests that it is of some central importance in the pathogenesis of these tumors.

I look forward to continuing our interactions with this exciting series of experiments; good luck with them!

Sincerely,

Stephen P. Ethier, Ph.D.
Associate Professor and Director,
Division of Cancer Biology